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Development of cpSSR markers for analysis of genetic diversity in *Gladiolus* cultivars

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ABSTRACT

We report the development of 12 novel microsatellite (SSR) markers isolated from repeat sequences of chloroplast region of *Gladiolus*. A total of 1188 nucleotide sequences of chloroplast regions of *Gladiolus* were used to search for simple sequence repeats and 108 sequences were found to contain 124 SSRs. Dinucleotide repeat SSRs were most frequent (94.4%) with the AT/AT (59.7%) repeat motif followed by TA/TA (31.5%). Assessment of genetic diversity among 62 commercially important *Gladiolus* cultivars was carried out using 12 SSR markers. Out of 12 SSR markers, only six showed polymorphism with allelic variation ranging from 2 to 8. These six SSR markers revealed high genetic diversity (84.6%) across different *Gladiolus* cultivars. The average value of Polymorphic information content (PIC = 0.32) diversity index (2.03), expected heterozygosity (0.354) and Shannon's information index (0.522) were also significantly high. These markers could be utilized for future breeding programmes, genetic diversity studies, and germplasm management of this important ornamental crop.

1. Introduction

The genus *Gladiolus* comprises about 265 species mainly distributed in tropical Africa, Madagascar, Arabian Peninsula, the Mediterranean basin, Europe and Asia (Goldblatt and Manning, 1998). In India *Gladiolus* is primarily grown in the states like Uttar Pradesh, Uttarakhand, Himachal Pradesh, Haryana, Delhi, Karnataka, Punjab, West Bengal, Assam, Sikkim, and Meghalaya. *Gladiolus* is an economically important ornamental crop, cultivated for its multi-coloured spikes throughout the world. Beautiful inflorescence, a variety of colours and its long lasting shelf life have made it excellent for vase decoration, preparation of bouquets and other floral arrangements (Kumar and Raju, 2007). Among cut flowers, it ranks second in area and production in India (Kolavalli et al., 1991). Though, there are few studies reported on the assessment of genetic characterization in *Gladiolus* based on molecular markers such as RAPD, ISSR, DAMD and AFLP (Dallavalle et al., 2002; Jingang et al., 2006, 2008; Pragya et al., 2010; Ranjan et al., 2010; Raycheva et al., 2011; Nasir et al., 2012; Singh et al., 2016a), there are no reports available on the development and characterization of simple sequence repeats (SSRs) markers.

SSR markers also called microsatellites and are one of the most widely used molecular markers in plant breeding and genetic diversity studies (Powell et al., 1996a; Gupta and Varshney, 2000). They are 1–6 base tandem repeats of DNA sequences, present in coding and non-coding regions of prokaryotes and eukaryotes (Field and Wills, 1998). SSRs have preferred marker system due to their co-dominant inheritance, high reproducibility, high polymorphism and multi-allelic nature (Powell et al., 1996b; Gupta et al., 2003). Expressed Sequence Tags (EST-SSR) markers have preferred over genomic-SSR (gSSR) for several genetic improvement programmes due to their higher interspecific transferability (Guo et al., 2006). Chloroplast SSR (cpSSR) available in NCBI database is the easiest and cheapest source for SSR development. cpSSR markers are credited with various advantages including easy identification by electronic sorting, presence in gene-rich regions and high transferability across species and widely used to estimate genetic diversity of plants (Bryan et al., 1999; Ribeiro et al., 2002; Thiel et al., 2003; Takahashi et al., 2016). cpSSRs are promising for the study of cross-species transferability since the flanking regions are strongly conserved, universal primers can easily be developed (Weising and Gardner, 1999; Ebert and Peakall, 2009a). The aim of the present study was to utilize the chloroplast database of *Gladiolus* to develop cpSSR markers and to estimate the genetic diversity in *Gladiolus* cultivars.

Abbreviations: cpSSRs, Chloroplast SSR; gSSR, genic-SSR; EST-SSR, Expressed Sequence Tags; cpSSR, Chloroplast SSR; bp, Base pair; ib, band informativeness; TNR, trinucleotide repeats; UPGMA, Unweighted Pair Group Method with Arithmetic Mean; TF, total fragment; PF, polymorphic fragment; PPF, percentage polymorphic fragment; PIC, polymorphic information content; RP, resolving power; DI, diversity index; He, expected heterozygosity; I, Shannons information index; EMR, effective multiplex ratio; MI, marker index; SSR, Simple Sequence Repeats

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2. Materials and methods

2.1. Plant materials and DNA isolation

Young leaves from 62 *Gladiolus* cultivars, which are grown at experimental plot of CSIR- National Botanical Research Institute, Lucknow (CSIR-NBRI) India were collected for the genomic DNA isolation (Table S1). High quality genomic DNA was isolated from the fresh leaf tissues following CTAB method (Doyle and Doyle, 1990). Quality and quantity of the genomic DNA were measured by gel electrophoresis on 0.8% agarose gel, stained with ethidium bromide, and compared with a set of known DNA concentration standards (EcoRI Hind III), and by UV spectroscopy using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc. USA).

2.2. Data mining and cpSSR identification

The nucleotide sequences mostly cpDNA was available in the NCBI database (www.ncbi.nlm.nih.gov. March 11, 2015) for *Gladiolus* (1188 nucleotide sequences) were retrieved in FASTA format. The presence of SSRs within these sequences was detected using SSRIT (simple sequence repeat identification tool) software (http://www.gramene.org/db/searches/ssrtool) with a minimum number of repeats of 5 for dinucleotide (DNR) and trinucleotide (TNR) and of 4 for tetranucleotide repeats.

2.3. Primer design and PCR amplification

The cpSSR-containing sequences were used to design flanking primers with WebSat software (Martins et al., 2009) because it offers the unique ability to visualise the cpSSR containing sequences. This feature ensured that the primer sequence did not overlap with the SSR of interest and that no primer pair contained more than one cpSSR. The major parameters selected for primer design were a primer length of 18 to 25 bp (optimum 22 bp), a PCR product size varying between 206 and 397 bp, GC content between 40% and 80%, and an optimum melting temperature of 60 °C. The short listing of primers pairs were done based on the repetitive motifs of cpSSR containing sequences.

Twelve pairs of SSR primers were synthesized from Sigma-Aldrich Pvt. Ltd. India. Six primer pairs were selected on the basis of their polymorphism and discrete fragment patterns. Amplification of microsatellite repeats were carried out in a 20 µL reaction mixture containing 2 × Phusion master mix with HF buffer (Thermo Scientific), 10 pmol each of forward and reverse primers and 20 ng genomic DNA. The amplification was performed in a Proflex PCR system (Applied Biosystem Life Technologies) programmed at an initial denaturation for 5 min at 95 °C followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 45 s at 55 °C–66 °C (Primer-specific) and extension for 1 min at 72 °C followed by final extension for 7 min at 72 °C.

The PCR products were resolved on 2.5% SFR™ agarose gel in 1 × TBE buffer stained with ethidium bromide at 60 V for 5 to 6 h and the fragments were visualized and documented in UV Tech Gel Documentation System (UK). The fragment size of the amplified products was compared with 100 bp DNA ladder. The gel profiles were photographed and stored as digital pictures in gel documentation system.

2.4. Data analysis

The data for SSR markers were scored manually as '1' (presence of band) and '0' (absence band). The polymorphic information content (PIC) for each primer was calculated according to Botstein et al. (1980). A pair wise matrix of similarity between genotypes was determined for the band data using Jaccard's similarity coefficient for UPGMA method and dendrogram was constructed in the FreeTree program (ver. 0.9.1.5) (Pavlicek et al., 1999). The comparative statistical analysis was carried out to determine the utility of each marker considered in the present study. The diversity index (DI), the effective multiplex ratio (EMR) and the marker index (MI) were calculated according to Powell et al. (1996a, 1996b). Resolving power (RP) of each primer was calculated as per Prevost and Wilkinson (1999): $RP = \Sigma I_b$, where I_b is the band informativeness and calculated as $I_b = 1 - [2x (0.5 - p)]$ where p is the proportion of the genotypes containing the band. Expected heterozygosity and Shannon's information index were computed by using GenAlex version 6.5 (Peakall and Smouse, 2012). Principal co-ordinate analysis (PCOA) was performed using the NTSYS version 2.01e software (Rohlf, 2000).

Table 1
Details of cp-sequences and SSRs identified in *Gladiolus*.

Parameters	Numbers
Total number of sequences examined	1188
Total size of examined sequences (bp)	88,837
Total number of identified SSRs	124
Number of SSR containing sequences	108
Number of sequences containing > 1 SSR	16
Number of Primer designed	12

3. Results

3.1. Frequency and distribution of cpSSRs

A total of 1188 nucleotide sequences varying in length from 441 to 2393 bases (average 822) were downloaded and assembled (Table 1). Of the 1188 sequences, 108 were found to contain 124 SSRs. Of the 108 SSR-containing sequences, 16 (15%) contained > 1 SSR. Among the type of SSRs, the highest proportion was represented by dinucleotide repeat (94.4%) followed by trinucleotide (TNR) (6.6%) repeats, no tetranucleotide, pentanucleotide and hexanucleotide SSR were identified under the criteria used for the SSR search. The majority of SSR motifs were smaller repeat length contains < 10 repeats and only one was found to contain 21 repeats. The most common type of SSR motif was AT/AT (59.7%) repeat motif followed by TA/TA (31.5%), GA/TC (2.4%) AAG/CTT (1.6%) (Table S2). A total of 12 cpSSR markers were developed (Table 2).

3.2. Polymorphism analysis

Microsatellite (SSR) markers were developed and characterized for the *Gladiolus* cultivars during the present investigation. Out of the 12 cpSSR markers, six cpSSR markers were used to characterize and estimate the genetic diversity in 62 *Gladiolus* cultivars based on clear and discrete banding patterns. These SSR primers generated a total number of 37 discrete and scorable fragments. Out of the 37 fragments, 32 (84.6%) were found to be polymorphic and 5 (15.4%) as monomorphic in nature. The number of fragments produced by each SSR primer ranged from 3 (G10SSR) to 9 (G8SSR and G9SSR). The percentage of polymorphism varied from 66.6% (G10SSR) to 100% (G5SSR and G7SSR). The Polymorphic information content (PIC) value ranged from 0.28 (G5SSR and G7SSR) to 0.41 (G8SSR) with an average value of 0.32. The resolving power (RP) was found to be highest (12) in G9SSR, whereas corresponding lowest value (4) was obtained in the case of G11SSR with an average RP value 6.8 per primer. Diversity index ranged from 0.87 (G10SSR) to 3.61 (G8SSR) with an average value of 2.03. Expected heterozygosity ranged from 0.260 (G10SSR) to 0.426 (G9SSR) with an average of 0.354 and Shannon's information index from 0.375 (G10SSR) to 0.607 (G9SSR) with an average of 0.522. Effective multiplex ratio (EMR) and Marker index (MI) values for SSR markers were 27.6 and 10.69, respectively (Table S3).